

This page Is Inserted by IFW Operations
And is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of
The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
Please do not report the images to the
Image Problem Mailbox.**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : A61K 39/395, C07K 15/28, C12N 15/13, C12P 21/08	A1	(11) International Publication Number: WO 94/12214 (43) International Publication Date: 9 June 1994 (09.06.94)
(21) International Application Number: PCT/US93/11611 (22) International Filing Date: 30 November 1993 (30.11.93) (30) Priority Data: 07/983,949 1 December 1992 (01.12.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/983,949 (CIP) Filed on 1 December 1992 (01.12.92) (71) Applicant (for all designated States except US): PROTEIN DESIGN LABS, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94303 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CO, Man, Sung [GB/US]; 10230 Yoshino Place, Cupertino, CA 95014 (US). LAN- DOLFI, Nicholas, F. [US/US]; 246 Seaside Drive, Milpitas, CA 95035 (US). (74) Agents: DUNN, Tracy, J. et al.; Townsend and Townsend Khourie and Crew, One Market Plaza, 20th floor, Steuart Street Tower, San Francisco, CA 94105 (US).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: HUMANIZED ANTIBODIES REACTIVE WITH CD18 (57) Abstract Humanized immunoglobulins specifically reactive with CD18 are prepared employing recombinant DNA technology for use in e.g., treatment of inflammatory disorders.		

A02

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

HUMANIZED ANTIBODIES REACTIVE WITH CD185 Field of the Invention

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel biologics and, more particularly, for example, to the production of non-
10 immunogenic (in humans) immunoglobulins specific for the CD18 protein and their uses in vitro and in vivo.

Background of the Invention

The ability of cells to adhere to one another plays
15 a critical role in development, normal physiology, and disease processes. This ability is mediated by adhesion molecules, generally glycoproteins, expressed on cell membranes. Often, an adhesion molecule on one cell type will bind to another adhesion molecule expressed on a different
20 cell type, forming a receptor counter-receptor pair. Three very important classes of adhesion molecules are the integrins, selectins, and immunoglobulin (Ig) superfamily members (see Springer, Nature 346, 425 (1990); Osborn, Cell 62, 3 (1990); Hynes, Cell 69, 11 (1992), all of which are
25 incorporated herein by reference). These molecules are especially vital to the interaction of leukocytes and platelets with themselves and with the extracellular matrix and vascular endothelium.

Integrins are heterodimeric transmembrane
30 glycoproteins consisting of an α chain (120-180 kD) and a β chain (90-110 kD), generally having short cytoplasmic domains. The α subunits all share sequence homology and motifs with each other, as do the β subunits. The three known integrins containing the β subunit designated β_2 are
35 important to the function of T cells, neutrophils and monocytes. LFA-1 ($\alpha_L\beta_2$) is widely distributed on lymphocytes, granulocytes and monocytes. Its counter-receptor is ICAM-1 (and perhaps of lesser importance, ICAM-2) an Ig family molecule which is expressed on many cells including

leukocytes and is up-regulated on vascular endothelium by cytokines such as TNF and IL-1. Blocking LFA-1 on T cells with antibodies to either the α or β subunit strongly inhibits adhesion-dependent functions such as CTL-mediated lysis of target cells. Mac-1 ($\alpha_M\beta_2$) is distributed on neutrophils and monocytes, and its counter-receptor is also ICAM-1 (and possibly ICAM-2). Among other things, Mac-1 is the type 3 complement receptor (CR3) and binds the C3bi fragment. The third β_2 integrin, P150,95 ($\alpha_X\beta_2$), is also found on neutrophils and monocytes, but seems of less importance. The α subunits of LFA-1, Mac-1 and P150,95 are also given the respective CD designations CD11a, CD11b and CD11c, while β_2 is also denoted CD18, so that LFA-1 is CD11a/CD18 and Mac-1 is CD11b/CD18.

There are three known selectins, which were previously known as LECCAMS, and are now designated L-selectin (also called LECAM-1, Mel-14 or LAM-1), E-selectin (also called ELAM-1) and P-selectin (also called GMP140 or PADGEM). They have all been sequenced at the cDNA level and share sequence homology and motifs, including a lectin-like domain. L-selectin has a dual role: it is a homing receptor on T cells for the high endothelial venules of peripheral lymph nodes, and it is an adhesion molecule on neutrophils for endothelium (Hallmann et al., Biochem. Biophys. Res. Commun. 174, 236 (1991), which is incorporated herein by reference). E-selectin and P-selectin are both induced on endothelium by cytokines, although with different kinetics. L-selectin is a counter-receptor on neutrophils for both E-selectin and P-selectin (Picker et al., Cell 66, 921 (1991), which is incorporated herein by reference), although all three selectins probably have other counter-receptors as well. In particular, E-selectin binds the carbohydrate group sialyl Lewis x (sLex) (Lowe et al., Cell 63, 475 (1990)), which is incorporated herein by reference), and while this carbohydrate is prominently presented on L-selectin (Picker et al., Cell 66, 921 (1991)), it may occur on other proteins as well. E-selectin is expressed especially in cutaneous sites of inflammation and also serves as an adhesion molecule

for skin-homing T cells that may contribute to the inflammation (Picker et al. Nature 349, 796 (1991), which is incorporated herein by reference).

5 In various assays, antibodies to CD11a, CD11b, CD18, L-selectin and E-selectin all block binding of neutrophils to activated endothelial cells to a lesser or greater degree, but the most complete inhibition is generally achieved by the combination of an antibody to CD18 and an antibody to L- or E-selectin (see e.g., Luscinskas, J. Immunol. 142, 2257 (1989), which is incorporated herein by reference). A recent but now widely accepted model accounts for these facts with a three step process of adhesion (Butcher, Cell 67, 1033 (1991), which is incorporated herein by reference). In the first step, neutrophils reversibly bind to inflamed vascular endothelium via the selectins, which bind well under conditions of flow, causing the neutrophils to literally roll along the vascular wall. The neutrophils are then activated by a variety of stimulants surrounding or released by the endothelium, including IL-8, PAF and C5a. The activated neutrophils shed L-selectin and up-regulate Mac-1. In the final step, binding of Mac-1 to ICAM-1 and perhaps other counter-receptors on the endothelial cells allows stable adhesion and extravasation through the endothelium.

25 Although important for eliminating infection, neutrophils are now believed to be a major cause of tissue damage during inflammation by binding to vascular endothelium and migrating through it into tissues (Harlan, Acta. Med. Scand. Suppl. 715, 123 (1987), which is incorporated herein by reference). The neutrophils release proteases and toxic, reactive oxygen metabolites which damage endothelium as well as other tissues. In principle, antibodies or other antagonists of the integrin and selectin adhesion molecules could abort this process, by preventing neutrophils from binding to endothelium and from extravasating into tissues. Hence such antibodies could be used to treat a great many different disease conditions of which inflammation is an important component.

For example, in animal models anti-CD18 antibodies, which bind to both LFA-1 and Mac-1, have been especially useful in reducing ischemia-reperfusion injury (see, e.g., Vedder et al., J. Clin. Invest. 81, 939 (1988); Vedder et al., Proc. Natl. Acad. Sci. USA 87, 2643 (1990); U.S. Patent No. 4,797,277). They also reduce neutrophil-mediated damage in the lung in response to various insults (Doerschuk et al., J. Immunol. 144, 2327 (1990) and Mulligan et al., J. Immunol. 148, 1847 (1992)), including gram-negative sepsis (Walsh et al., Surgery 110, 205 (1991)). In a rabbit model, anti-CD18 antibodies also protect from lethality due to meningitis (Tuomanen et al., J. Exp. Med. 170, 959 (1990)). They may also be useful in preventing or treating organ transplant rejection because they block T-cell function.

For example, injection of antibodies to L-selectin or E-selectin into rodents suppressed neutrophil accumulation within inflamed peritoneum (Jutila et al. J. Immunol. 143, 3318 (1989) and Mulligan et al., J. Clin. Invest. 88, 1396 (1991)). Intravital video microscopy was used to show that an anti-L-selectin antibody strongly inhibits rolling of leukocytes along the vascular wall endothelium of mesenteric venules exteriorized from rabbits (von Andrian et al., Proc. Natl. Acad. Sci. USA 88, 7538 (1991)). An anti-E-selectin antibody greatly reduced vascular injury induced by immune complex deposition in the skin or lungs of rats, and substantially reduced neutrophil accumulation at those sites (Mulligen et al., J. Clin. Invest. 88, 1396 (1991)). Also, in a primate model of extrinsic asthma, an anti-E-selectin antibody greatly reduced neutrophil influx into the lung and associated late-phase airway obstruction after antigen inhalation (Gundel et al., J. Clin. Invest. 88, 1407 (1991)).

The antibody NA-8 has been developed that binds to human CD18 as disclosed herein. This antibody partially or completely blocks the binding of human neutrophils to stimulated human umbilical vein endothelial cells. Because of its ability to block binding of neutrophils to endothelial cells, this antibody could be used to treat inflammatory disease conditions (see above).

Unfortunately, the use of non-human monoclonal antibodies such as NA-8 have certain drawbacks in human treatment, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, have a relatively short circulating half-life, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against an antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, as increasing numbers of different mouse or other antigenic (to humans) monoclonal antibodies can be expected to be developed to treat various diseases, after the first or several treatments with any different non-human antibodies, subsequent treatments even for unrelated therapies can be ineffective or even dangerous in themselves, because of cross-reactivity.

While the production of so-called "chimeric antibodies" (e.g., mouse variable regions joined to human constant regions) has proven somewhat successful, a significant immunogenicity problem remains. In general, the production of human immunoglobulins reactive with CD18 antigen, as with many antigens, would be extremely difficult using typical human monoclonal antibody production techniques. Similarly, utilizing recombinant DNA technology to produce so-called "humanized" or "reshaped" antibodies (see, e.g., Riechmann et al., Nature 332, 323 (1988) and EPO Publication No. 0239400, which are incorporated herein by reference), provides uncertain results, in part due to unpredictable binding affinities of the resultant immunoglobulins.

Thus, there is a need for improved forms of humanized immunoglobulins specific for CD18 antigen that are substantially non-immunogenic in humans, yet easily and

economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

5 Summary of the Invention

 The present invention provides novel compositions useful, for example, in the treatment of inflammatory human disorders, the compositions containing mouse or humanized immunoglobulins specifically capable of binding to CD18. The immunoglobulins can have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions functionally joined to human framework region segments. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to the CD18 at affinity levels stronger than about 10^7 M^{-1} . These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to CD18.

 The immunoglobulins, including binding fragments and other derivatives thereof, of the present invention may be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for humanized immunoglobulin framework regions and a second sequence set coding for the desired immunoglobulin complementarity determining regions can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

 The humanized immunoglobulins may be utilized alone in substantially pure form, or together with a chemotherapeutic agent such as a non-steroidal anti-inflammatory drug, a corticosteroid, or an immunosuppressant. All of these compounds will be particularly useful in treating inflammatory disorders. The humanized immunoglobulins or their complexes can be prepared in a

pharmaceutically acceptable dosage form, which will vary depending on the mode of administration.

BRIEF DESCRIPTION OF THE FIGURES

5 Fig. 1. Sequences of the cDNA and translated amino acid sequences of the light chain (A) [SEQ. ID NOS:1 and 2] and heavy chain (B) [SEQ. ID NOS:3 and 4] variable regions of the NA-8 antibody. The mature light chain begins with amino acid 21 D, and the mature heavy chain begins with amino acid
10 20 Q, preceded by the respective signal sequences.

Fig. 2. Amino acid sequences of the mature light chain (A) [SEQ. ID NO:5] and heavy chain (B, [SEQ. ID NO:6] C [SEQ. ID NO:7]) variable regions of the mouse NA-8 antibody (upper lines) and humanized NA-8 antibody (B; lower lines)
15 and humanized NA-8a antibody (C, lower lines). The three CDRs in each chain are underlined. Residues in the framework that have been replaced with mouse amino acids or typical human amino acids in the humanized antibody are double underlined.

20 Fig. 3. Nucleotide sequences of the genes encoding the light chain (A) [SEQ. ID NOS:8 and 9] and heavy chain (B) [SEQ. ID NOS:10 and 11] variable regions of the humanized NA-8 antibody, beginning and ending with the XbaI sites, and translated amino acid sequences, including signal sequences.

25 Fig. 4. Competitive binding of mouse and humanized IgG1 and IgG4 NA-8 antibodies. The target cells were THP-1 cells, a human monocytic line that expresses human CD18 (ATCC TIB 202). 2×10^5 cells were incubated with 3 ng of ^{125}I -labeled tracer mouse antibody ($2 \mu\text{Ci}/\mu\text{g}$), together with
30 increasing amounts of mouse or humanized competitor antibody as indicated in 0.2 ml of binding buffer (PBS + 2% FBS + 0.1% azide) for 1 hr at 4°C. Cells were washed and pelleted, and their bound radioactivity measured. The concentrations of bound and free tracer antibody were calculated.

35 Fig. 5. Binding of human neutrophils to IL-1 stimulated human umbilical cord endothelial cells (HUVEC). The neutrophils were first treated with irrelevant control

antibody, mouse NA-8 antibody, or humanized IgG1 NA-8 antibody, as indicated.

Definitions

5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can
10 be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

15 The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man
20 in the laboratory is naturally-occurring.

 As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially
25 purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the
30 composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

35 As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by

marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., ^3H , ^{14}C , ^{32}P , ^{35}S , ^{125}I , ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

DETAILED DESCRIPTION OF THE INVENTION

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, cell culture, and transgene incorporation (e.g., electroporation, microinjection, lipofection). Generally enzymatic reactions, oligonucleotide synthesis, and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

In accordance with the present invention, mouse or humanized immunoglobulins specifically reactive with CD18 related epitopes are provided. These immunoglobulins, which

have binding affinities to CD18 under suitable binding conditions (e.g., physiological serum conditions) of at least about 10^7 M^{-1} , and preferably 10^8 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., binding to CD18-expressing cells, such as neutrophils. The humanized immunoglobulins will have a human framework and will have one or more complementarity determining regions (CDRs) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with CD18. In a preferred embodiment, one or more of the CDRs will come from the NA-8 antibody, and the humanized immunoglobulin will be of the IgG1 or IgG4 isotype. Thus, the immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of adhesion-related pathological conditions (e.g., inflammatory disorders) in human patients by a variety of techniques.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The NH_2 -terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH part of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (See, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pgs. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference.)

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the same general structure of relatively conserved framework regions joined by three hypervariable regions, also called

Complementarity Determining Regions or CDRs (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1987); and Chothia and Lesk, J. Mol. Biol., 196, 901-917 (1987), which are incorporated herein by reference). The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies; including, for example, Fv, Fab, and (Fab')₂ as well as bifunctional hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science, 242, 423-426 (1988), which are incorporated herein by reference). (See, generally, Hood et al., Immunology, Benjamin, N.Y., 2nd ed. (1984), Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and Hunkapiller and Hood, Nature, 323, 15-16 (1986), which are incorporated herein by reference).

It is well known that native forms of "mature" immunoglobulins will vary somewhat in terms of length by deletions, substitutions, insertions or additions of one or more amino acids in the sequences. Thus, both the variable and constant regions are subject to substantial natural modification, yet are "substantially identical" and still capable of retaining their respective activities. Human constant region and rearranged variable region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells. Similar methods can be used to isolate nonhuman immunoglobulin sequences from non-human sources. Suitable source cells for the DNA sequences and host cells for

expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated
5 herein by reference).

In addition to these naturally-occurring forms of immunoglobulin chains, "substantially identical" modified heavy and light chains can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the
10 chains can vary from the naturally-occurring sequence at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Alternatively, polypeptide fragments comprising only a
15 portion of the primary structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., binding activity). In particular, it is noted that like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct
20 biological activities. In general, modifications of the genes encoding the desired epitope binding components may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and Roberts, S. et al., Nature 328:731-
25 734 (1987), both of which are incorporated herein by reference). In preferred embodiments of the invention, the epitope binding component is encoded by immunoglobulin genes that are "chimeric" or "humanized" (see, generally, Co and Queen (1991) Nature 351:501, which is incorporated herein by
30 reference). Generally, the humanized antibodies of the invention will comprise heavy chain variable region sequences wherein the sequence of the humanized immunoglobulin heavy chain variable region framework is more than 65% identical but less than 95% identical to the sequence of the donor
35 immunoglobulin heavy chain variable region framework, preferably the variable region framework is less than 10 % identical to the sequence of the donor immunoglobulin.

Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as γ_1 and γ_4 . A typical therapeutic chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody, although other mammalian species may be used.

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (i.e., other than the CDRs) among different immunoglobulins in a single species, as defined by Kabat, et al., op. cit. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or more) to the framework region of a naturally occurring human antibody.

As used herein, the term "humanized immunoglobulin" refers to an immunoglobulin comprising a human framework, at least one CDR from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, i.e., at least about 85-90%, preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. For example, a humanized immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody.

Humanized antibodies have at least three potential advantages over mouse and in some cases chimeric antibodies for use in human therapy:

- 1) because the effector portion is human, it may interact better with the other parts of the human immune system (e.g., destroy the target cells more efficiently by complement-dependent

cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).

2) The human immune system should not recognize the framework or C region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.

3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (Shaw, D. et al., J. Immunol. 138, 4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life more like that of naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDRs from an immunoglobulin capable of binding to a desired epitope of CD18, such as monoclonal antibody NA-8.

The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human framework regions. Exemplary DNA sequences, which on expression code for the polypeptide chains comprising the heavy and light chain CDRs of monoclonal antibody NA-8 are included in Fig.

1. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below. For a detailed description of the design and production of humanized immunoglobulins, see, commonly assigned serial nos. 07/290,975 and 07/310,252, filed December 28, 1988 and February 13, 1989, respectively, both of which are incorporated herein by reference.

The DNA segments will typically further include an expression control DNA sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions.

5 Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate
10 host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow.

15 The nucleic acid sequences of the present invention capable of ultimately expressing the desired humanized antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C
20 regions), as well as by a variety of different techniques. Joining appropriate genomic and synthetic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and Reichmann, L. et al., Nature 332,
25 323-327 (1988), both of which are incorporated herein by reference).

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat
30 op. cit. and WP87/02671). The CDRs for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to CD18 and produced in any convenient mammalian source, including, mice, rats, rabbits, or other vertebrate capable of producing
35 antibodies by well known methods. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection

(Catalogue of Cell Lines and Hybridomas, Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference). In preferred embodiments, the CDRs have sequences corresponding to the CDR sequences of NA-8, and may include degenerate nucleotide sequences encoding the corresponding CDR amino acid sequence(s) of NA-8.

In addition to the humanized immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can vary from the native sequences at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the humanized immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8, 81-97 (1979) and Roberts S. et al, Nature 328, 731-734 (1987), both of which are incorporated herein by reference).

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., binding activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in the vectors pVk and pVg1-dhfr using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce (Fab')₂ fragments. Single chain antibodies may be produced by joining VL and VH with a DNA linker (see Huston et al., op cit., and Bird et al., op cit.). As one example, Fv or Fab fragments may be produced in E. coli according to the methods of Buchner and Rudolph (1991) Bio/Technology 9: 157-162 and Skerra et al. (1991) Bio/Technology 9: 273-277, incorporated herein by reference). Fv and Fab may also be produced by

expression of encoding polynucleotides in eukaryotic, preferably mammalian, cells. Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes (e.g., enzymes, see, commonly assigned U.S.S.N. 132,387, filed Dec. 15, 1987, which is incorporated herein by reference) to produce fusion proteins (e.g., immunotoxins) having novel properties.

Expression of the humanized immunoglobulin sequences in bacterial hosts may be used to advantage to select higher affinity humanized immunoglobulin sequences by mutagenizing the CDR regions and producing bacteriophage display libraries which may be screened for humanized immunoglobulin CDR variants which possess high affinity and/or high specificity binding to CD18. One potential advantage of such affinity sharpening is the generation of humanized immunoglobulin CDR variants which have improved binding affinity and/or reduced cross-reactivity with molecules other than CD18s. Methods for producing phage display libraries having immunoglobulin variable region sequences are provided in the art, for example see Cesareni (1992) FEBS Lett 307: 66-70; Swimmer et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 3756-60; Gram et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 3576-80; Clackson et al. (1991) Nature 352: 624-8; Scott and Smith (1990) Science 249: 386-90; Garrard et al. (1991) Bio/Techniques 9: 1373-1377, which are incorporated herein by reference. The resultant affinity sharpened CDR variant humanized immunoglobulin sequences are subsequently expressed in a suitable host for efficient expression.

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers,

e.g., tetracycline-resistance (tet^R), G418-resistance (neo^R), mycophenolic acid-resistance (gpt), or HSV-tk, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

Plants and plant cell cultures may be used for expression of the humanized immunoglobulins of the invention. (Larrick and Fry (1991) Hum. Antibodies Hybridomas 2(4): 172-89; Benvenuto et al. (1991) Plant Mol. Biol. 17 (4): 865-74; Durin et al. (1990) Plant Mol. Biol. 15(2): 281-93; Hiatt et al. (1989) Nature 342: 76-8, incorporated herein by reference). Preferable plant hosts include, for example: Arabidopsis, Nicotiana tabacum, Nicotiana rustica, and Solanum tuberosum. A preferred expression cassette for expressing polynucleotide sequences encoding the humanized anti-CD18 antibodies of the invention is the plasmid pMOG18

in which the inserted polynucleotide sequence encoding the humanized immunoglobulin chain is operably linked to a CaMV 35S promoter with a duplicated enhancer; pMOG18 is used according to the method of Sijmons et al. (1990)

5 Bio/Technology 8: 217-221, incorporated herein by reference. Alternatively, a preferred embodiment for the expression of humanized immunoglobulins in plants follows the methods of Hiatt et al. (1989) op.cit., with the substitution of polynucleotide sequences encoding the humanized anti-CD18
10 antibodies of the invention for the immunoglobulin sequences used by Hiatt et al. Agrobacterium tumifaciens T-DNA-based vectors may also be used for expressing humanized immunoglobulin sequences, preferably such vectors include a marker gene encoding spectinomycin-resistance or other
15 selectable marker.

Insect cell culture may also be used to produce the humanized immunoglobulins of the invention, typically using a baculovirus-based expression system. The humanized immunoglobulins may be produced by expressing polynucleotide
20 sequences encoding the humanized immunoglobulins according to the methods of Putlitz et al. (1990) Bio/Technology 8: 651-654, incorporated herein by reference. The method of Putlitz et al. can be followed with the modification that polynucleotide sequences encoding the humanized anti-CD18
25 antibodies of the invention are inserted in place of the mouse monoclonal Ab 6A4 heavy chain and light chain cDNA sequences of Putlitz et al.

In addition to microorganisms and plants, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Mammalian cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have
30 been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines, etc, or transformed B-cells or hybridomas. Expression
35 vectors for these cells can include expression control

sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., Immunol. Rev. 89, 49-68 (1986), which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, cytomegalovirus and the like. Generally, a selectable marker, such as a neo^R expression cassette, is included in the expression vector.

Transgenes encoding a humanized immunoglobulin of the invention may be used to generate transgenic nonhuman animals which express the desired humanized immunoglobulin, typically in a recoverable body fluid such as milk or serum. Such transgenes comprise a polynucleotide sequence encoding the humanized immunoglobulin(s) operably linked to a promoter, usually with a linked enhancer, such as a rodent immunoglobulin enhancer or a casein gene promoter/enhancer (Buhler et al. (1990) Bio/Technology 8: 140-143; Meade et al. (1990) Bio/Technology 8: 443-446, incorporated herein by reference). Transgenes may be transferred into cells and embryos according to the methods described in the art and infra for homologous recombination constructs. Preferred nonhuman animals include: mice, rats, sheep, cows, and goats; with expression in bovine milk being particularly preferred. Purification of the humanized antibodies is accomplished by art-known purification methods for immunoglobulin purification.

Homologous recombination constructs may also be used for producing cells or transgenic animals expressing the humanized immunoglobulins of the invention. Preferred promoter/enhancer combinations for operable linkage to polynucleotide sequences encoding the humanized immunoglobulins of the invention in the homologous recombination constructs include those associated with the albumin gene, β -casein gene, α -casein gene, whey protein genes, or lactalbumin gene. Most usually, a selectable marker gene expression cassette (e.g., neo^R, HSV-tk, or gpt

operably linked to a constitutive promoter such as the pgk gene promoter) is included in the homologous recombination construct for selection. The homologous targeting constructs encoding humanized immunoglobulins of the invention can be transferred into embryonic stem (ES) cells by lipofection, electroporation, or needle microinjection, or into fertilized animal embryos by pronuclear needle microinjection, or other art-accepted method. If ES cells are used for transgenesis, the resultant ES cells are usually selected by positive selection, and optionally be negative selection as well, and verified for correctly targeted recombination by PCR or Southern blotting. Correctly targeted ES cells are incorporated into blastocysts by blastocyst injection according to methods known in the art and cited herein. General principles regarding the construction of targeting constructs and selection methods are reviewed in Bradley et al. (1992) Bio/Technology 10: 534, incorporated herein by reference.

Chimeric targeted mice are derived according to Hogan, et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed., IRL Press, Washington, D.C., (1987) which are incorporated herein by reference. Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed., IRL Press, Washington, D.C. (1987); Zijlstra et al., Nature 342:435-438 (1989); and Schwartzberg et al., Science 246:799-803 (1989), each of which is incorporated herein by reference).

Frequently, homologous targeting constructs are propagated in cloning hosts (e.g., bacteria, yeast) and may also include an origin of replication and selectable marker(s) for facile cloning.

It is frequently preferable to use a transfection technique with linearized transgenes containing only modified target gene sequence(s) and without vector sequences. The modified gene site is such that a homologous recombinant

between the exogenous targeting construct and the endogenous DNA target sequence can be identified by using carefully chosen primers and PCR or by Southern blot analysis, followed by analysis to detect if PCR products or Southern blot bands specific to the desired targeted event are present (Erlich et al., (1991) Science 252: 1643, which is incorporated herein by reference). Several studies have already used PCR to successfully identify the desired transfected cell lines (Zimmer and Gruss (1989) Nature 338: 150; Mouellic et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 4712; Shesely et al. (1991) Proc. Natl. Acad. Sci. USA 88: 4294, which are incorporated herein by reference). This approach is very effective when the number of cells receiving exogenous targeting transgene(s) is high (i.e., with electroporation or with liposomes) and the treated cell populations are allowed to expand (Capecchi, M. (1989) Science 244:1288, incorporated herein by reference).

For making transgenic non-human animals (which include homologously targeted non-human animals), embryonal stem cells (ES cells) are preferred. Murine ES cells, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley, Cell 62:1073-1085 (1990)) essentially as described (Robertson, E.J. (1987) in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL Press), p. 71-112) may be used for homologous gene targeting. Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) Nature 326: 292-295), the D3 line (Doetschman et al. (1985) J. Embryol. Exp. Morph. 87: 27-45), and the CCE line (Robertson et al. (1986) Nature 323: 445-448). The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotency of the ES cells (i.e., their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal). The blastocysts containing the injected ES cells are allowed to develop in the uteri of pseudopregnant nonhuman females and are born as chimeric mice. The resultant transgenic mice are chimeric

for cells having the homologously integrated humanized immunoglobulin gene sequence(s) and are backcrossed and screened for the presence of the correctly targeted transgene(s) by PCR or Southern blot analysis on tail biopsy DNA of offspring so as to identify transgenic mice heterozygous for the inactivated lymphocyte transduction locus/loci. By performing the appropriate crosses, it is possible to produce, if desired, a transgenic nonhuman animal homozygous for multiple transgenes encoding a humanized immunoglobulin of the invention.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, lipofection, biolistics, viral-based transduction, or electroporation may be used for other cellular hosts. Tungsten particle ballistic transgenesis is preferred for plant cells and tissues. (See, generally, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982), which is incorporated herein by reference.)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982), which is incorporated herein by reference). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols.

I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

In a preferred embodiment, humanized immunoglobulins are produced which bind to CD18 with a binding affinity of at least $1 \times 10^7 \text{ M}^{-1}$ in standard binding conditions (e.g., phosphate-buffered saline with 2 percent fetal bovine serum at 25°C); one example of such humanized immunoglobulins is the humanized NA-8 antibody comprising the amino acid sequences shown in Fig. 2. The humanized antibodies of the invention preferably bind, in standard binding conditions, to human CD18 with an affinity of at least $1 \times 10^8 \text{ M}^{-1}$, more preferably with an affinity of at least $1 \times 10^9 \text{ M}^{-1}$, and advantageously with an affinity of at least $1 \times 10^{10} \text{ M}^{-1}$ or stronger.

The antibodies of the present invention will typically find use in the treatment of disease conditions with an inflammatory component, especially those which are mediated by neutrophils or T cells. A preferred application is the treatment of ischemia-reperfusion injury caused by myocardial infarction, cerebral ischemic event (e.g., stroke), renal infarction, brain surgery, shock, cardiac surgery (e.g., coronary artery bypass), elective angioplasty, and the like. Other preferred applications are the treatment of sepsis, adult respiratory distress syndrome, and multiple organ failure. The antibodies will find use in treating injury due to trauma, burns, frostbite or damage to the spinal cord. They will also find use in treating autoimmune diseases including by way of example and not limitation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, type I diabetes and uveitis, in treating inflammatory diseases of the skin such as psoriasis, and in treating meningitis and encephalitis. Other typical applications are the prevention and treatment of organ transplant rejection, graft-versus-host disease, and neoplasia.

Any humanized immunoglobulins of the present invention may also be used in combination with other antibodies, particularly humanized antibodies reactive with

different adhesion molecules. For example, suitable antigens to which a cocktail of humanized immunoglobulins may react include CD11a, CD11b, L-selectin, E-selectin, P-selectin and ICAM-1. Other suitable antigens are lymphokines such as IL-1, IL-2 and IFN- γ , and their receptors.

The humanized antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic agents. Typically, the agents may include non-steroidal anti-inflammatory drugs and corticosteroids, but numerous additional agents (e.g., cyclosporin) well-known to those skilled in the art of medicine may also be utilized. Indeed, the humanized immunoglobulins of the present invention will typically be used in combination with drugs currently used by those skilled in the art to treat particular diseases.

A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins to kill CD18 expressing cells. Immunotoxins are characterized by two components and are particularly useful for killing selected cells in vitro or in vivo. One component is a cytotoxic agent which is usually fatal to a cell when attached or absorbed. The second component, known as the "delivery vehicle," provides a means for delivering the toxic agent to a particular cell type, such as cells expressing a CD18 epitope. The two components are commonly chemically bonded together by any of a variety of well-known chemical procedures. For example, when the cytotoxic agent is a protein and the second component is an intact immunoglobulin, the linkage may be by way of heterobifunctional cross-linkers, e.g., SPDP, carbodiimide, glutaraldehyde, or the like. Production of various immunotoxins is well-known with the art, and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe et al., Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982), which is incorporated herein by reference. The components may also be linked genetically (see Chaudhary et

al., Nature 339, 394 (1989), incorporated herein by reference).

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents can include
5 radionuclides, such as Iodine-131 or other isotopes of iodine, Yttrium-90, Rhenium-188, and Bismuth-212 or other alpha emitters; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatin; and
10 cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain, etc., or an agent active at the cell surface, such as the phospholipase enzymes (e.g., phospholipase C). (See, generally, commonly assigned U.S.S.N. 07/290,968, "Chimeric Toxins," Olsnes and Phil,
15 Pharmac. There., 25, 355-381 (1982), and Monoclonal Antibodies for Cancer Detection and Therapy, eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985), all of which are incorporated herein by reference.)

The delivery component of the immunotoxin will
20 include the humanized immunoglobulins of the present invention. Intact immunoglobulins or their binding fragments, such as Fab or Fv, are preferably used. Typically, the antibodies in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant
25 regions may be utilized as desired.

For some applications, such as diagnostic uses, the humanized anti-CD-18 antibodies of the present invention will comprise a detectable label; typically the label is attached by covalent linkage or incorporation of a labeled
30 radionuclide into the glycoprotein immunoglobulin. For example and not limitation, one application of the humanized anti-CD-18 antibodies is as a research reagent and/or diagnostic reagent to detect quantitatively the presence of CD-18 on cell surfaces; frequently a labeled second antibody
35 (e.g., goat anti-human IgG) will be used to bind to the human sequence portion (e.g., the constant region and/or human variable region framework) of the humanized immunoglobulin. This approach can be used advantageously when the sample

being assayed for CD-18 expressing cells also contains other species which would interfere with a mouse primary antibody reactive with CD-18.

5 The humanized antibodies and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The humanized antibodies of the invention may also be administered, typically for local application, by gavage or lavage, intraperitoneal
10 injection, ophthalmic ointment, topical ointment, intracranial injection (typically into a brain ventricle), intrapericardial injection, or intrabursal injection. The compositions for parenteral administration will commonly comprise a solution of the immunoglobulin or a cocktail
15 thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, phosphate buffered saline (PBS), 0.4% saline, 0.3% glycine, human albumin solution and the like. These solutions are sterile and generally free of
20 particulate matter. These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium
25 acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.005%, usually at least about 1% to as much as 15 or
30 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

 Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered
35 water, and 1-10 mg of immunoglobulin. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions

will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference. Compositions suitable for lavage or other routes will be selected according to the particular use intended.

The antibodies of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present humanized antibodies or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from an inflammatory disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from about 1 to about 200 mg of antibody per dose, with dosages of from 5 to 100 mg per patient being more commonly used. Dosing schedules will vary with the disease state and status of the patient, and will typically range from a single bolus dosage or continuous infusion to multiple administrations per day (e.g., every 4-6 hours), or as indicated by the treating physician and the patient's condition. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the

minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present humanized immunoglobulins of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already suffering from a particular disease to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 1 to 50 mg per dose. Preferred prophylactic uses are for the prevention of adult respiratory distress syndrome in patients already suffering from sepsis or trauma; prevention of organ transplant rejection; and prevention of reperfusion injury in patients suffering from ischemia. In seriously ill patients, dosages of about 50 to 100 mg of humanized immunoglobulin per administration are frequently used, and larger dosages may be indicated.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

Humanized antibodies of the present invention can further find a wide variety of utilities in vitro. By way of example, the antibodies can be utilized for detection of CD18 antigens, for isolating specific leukocytes, or the like. For example but not for limitation, a humanized NA-8 immunoglobulin can be immobilized and contacted with blood extravasated from a patient to remove blood cells bearing CD18 antigens, and the remaining blood, depleted of CD18-bearing cells, may be reintroduced into the patient. Any residual humanized antibody present in the depleted blood reintroduced into the patient (e.g., as a consequence of

detachment from the immobilization support) would have reduced or negligible antigenicity as compared to a murine antibody.

For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the humanized antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme co-factors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

The following examples are offered by way of illustration, not by limitation. It will be understood that although the examples pertain to the NA-8 antibody, producing humanized antibodies with high binding affinity for CD18 may also be performed using CDRs from other monoclonal antibodies that bind to an epitope of CD18.

EXPERIMENTAL

Generation of anti-CD18 NA-8 antibody.

The mouse antibody NA-8 that binds to human CD18 was developed as follows. A mouse was subcutaneously immunized with 25×10^6 PMA-activated human neutrophils emulsified in Hunter's TiterMax™ adjuvant at two sites. The mouse was boosted 4 times by intraperitoneal injection of $20-25 \times 10^6$ PMA-activated neutrophils at intervals of 2 to 3 weeks. Four days following the final boost, the spleen cells were fused with myeloma cell to produce hybridomas, following methods well known in the art. Supernatants of hybridomas were then screened for the ability to inhibit neutrophil adherence to protein-coated plastic. Hybridoma supernatant samples (100 μ l) were transferred to a 96 well plate. A neutrophil suspension (100 μ l of 5×10^6 cells/ml) was added to each well. Following a 5 min incubation at room temperature, 20 μ l of a 500 ng/ml stock of PMA was added to

each well. The plate was incubated for thirty minutes at 37°C to allow the neutrophils to settle to the bottom of the well. Following this incubation, the wells of the plate were washed 3 times with RPMI to remove non-adherent cells. Each well was then stained with 50 µl of 0.2% crystal violet in 10% phosphate-buffered formalin for 20 min, then washed with H₂O and allowed to air dry. Wells in which neutrophils had adhered to the plastic were deeply stained, while wells in which the hybridoma supernatant inhibited the adherence did not stain and appeared clear. NA-8 was one of the hybridomas whose supernatant inhibited adherence.

That NA-8 specifically binds to CD18 was shown in two ways. Immunoprecipitation of labelled neutrophil lysate gave the same pattern as the Known anti-CD18 antibody IB4 (Wright et al. (1983) Proc. Natl. Acad. Sci. USA 80: 5699), that is bands of the correct size for CD18 and for co-precipitated CD11b and CD11c. Therefore, the antibody reacts with the CD11/CD18 complex, and the presence of 2 alpha chains (both CD11b and CD11c) implies that reactivity is in fact with CD18. Secondly, NA-8 binds to the same cell lines among a panel of cell lines as IB4. In particular, NA-8 reacts with Hut-102B and JURKAT cells as well as normal human peripheral blood lymphocytes, which express CD18 but not CD11b.

Cloning of heavy chain and light chain cDNA.

cDNAs for the heavy chain and light chain variable domain genes of NA-8 were cloned using anchored polymerase chain reactions as described (see Co. et al., J. Immunol. 148, 1149 (1992) and commonly assigned U.S.S.N. 07/634,278), using 3' primers that hybridized to the constant regions and contained HindIII sites, and 5' primers that hybridized to the dG tails and contained EcoRI sites. The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC18 vector for sequencing. For NA-8, at least two gamma-1 specific and two kappa specific clones were sequenced. The gamma-1 clones and the kappa clones are respectively identical in sequence. The cDNA variable domain

sequences and the deduced amino acid sequences are shown in Fig. 1.

Computer modeling of humanized antibodies.

5 In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (see, Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029 (1989) and WO 90/07861, which are incorporated herein by reference). The more homologous a human antibody is to the original murine antibody, the less likely will combining the murine CDRs with the human framework be to introduce distortions into the CDRs that could reduce affinity. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against the NBRF protein sequence database (performed with the MicroGenie Sequence Analysis Software (Beckman)), the antibody Eu was chosen to provide the framework sequences for humanization of NA-8.

10 The computer program ENCAD (M. Levitt, J. Mol. Biol. 168, 595 (1983), which is incorporated herein by reference) was used to construct a model of the NA-8 variable region. The model was used to determine the amino acids in the NA-8 framework that were close enough to the CDRs to potentially interact with them (category 4 below). To design the humanized light and heavy chain NA-8 variable regions, at each position the amino acid was chosen to be the same as in the Eu antibody, unless that position fell in one or more of four categories:

- 15 (1) The position fell within a CDR,
 (2) The Eu amino acid was unusual for human antibodies at that position, whereas the NA-8 amino acid was typical for human antibodies at that position,
20 (3) The position was immediately adjacent to a CDR,

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDRs).

For positions in these categories, the amino acid from the mouse NA-8 antibody was used.

The amino acids in each category are shown in Table 1. Some amino acids may be in more than one category. The final sequences of the humanized NA-8 light and heavy chain variable domains are shown in Fig. 2, A-B compared with the murine NA-8 sequences.

TABLE 1

<u>Category</u>	<u>Light Chain</u>	<u>Heavy Chain</u>
1	24-40, 56-62, 95-103	31-35, 50-66, 99-106
2	54, 87, 112	93, 95, 98, 107-109, 111
3	--	30, 67, 98, 107
4	22, 54, 69, 76	27, 30, 38, 48, 67-68, 70, 74

In addition, the amino acid from the mouse NA-8 antibody was chosen at light chain position 49, because it was at the light-heavy chain interface.

To determine whether amino acids 67, 68, 70 and 74 in the heavy chain variable region framework of humanized NA-8 were important in maintaining binding affinity, a second humanized version of NA-8 was constructed, designated humanized NA-8a. This antibody has the same light chain as the first version (Fig. 2A), but in the heavy chain the human EU amino acids were retained at positions 67, 68, 70 and 74, rather than being replaced by the murine NA-8 amino acids. The sequence of the mature heavy chain variable region of humanized NA-8a is shown in Fig. 2C. Humanized NA-8a was produced and characterized analogously to humanized NA-8 (see below).

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains,

including signal peptides, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences of the genes also included splice donor signals and an XbaI site at each end. For example, the nucleotide sequences and encoded humanized light and heavy chain variable domains of humanized NA-8 are shown in Fig. 3. Each gene was constructed from four overlapping synthetic oligonucleotides, as described (see, Co et al., J. Immunol. 148, 1149 (1992), and commonly assigned U.S.S.N. 07/634,278, which are incorporated herein by reference.) The heavy and light chain variable region genes were then respectively ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors (see, commonly assigned U.S.S.N. 07/634,278) in the appropriate orientations to produce the complete heavy and light chain genes. Reactions were carried out under conditions well-known in the art (Maniatis et al., op. cit.)

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Humanized NA-8 IgG1 antibody was then purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody was eluted with 0.2 M Glycine-HCl, pH3.0 and neutralized with 1 M Tris PH8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia), or by dialysis. To obtain cells producing higher levels of antibody, the transfected clones may be cultured in increasing concentrations of methotrexate.

To produce a humanized NA-8 antibody of the IgG4 isotype, another vector pVg4-dhfr was first constructed. To do so, the XbaI-BamHI fragment of pVg1-dhfr containing the γ 1 constant region was replaced with an approximately 2000 bp fragment of the human γ 4 constant region gene (Ellison and Hood, Proc. Natl. Acad. Sci. USA 79: 1984 (1982)) that extended from the HindIII site preceding the C_H1 exon of the

γ4 gene to 270 bp after the NsiI site following the C_H4 exon of the gene, using methods well-known to those skilled in the art, including polymerase chain reaction. The humanized NA-8 heavy chain variable region gene was then cloned into the XbaI site of pVg4-dhfr. This heavy chain plasmid was then transfected together with the above light chain plasmid into Sp2/0 cells, clones selected, and humanized NA-8 IgG4 antibody purified as described above for the IgG1 antibody. A humanized NA-8a IgG4 antibody is produced similarly.

Properties of humanized antibodies.

The affinity of the humanized NA-8 antibodies for CD18 were determined by competition with the radio-iodinated mouse NA-8 antibody (Fig. 4). The binding affinities were calculated according to the methods of Berzofsky (J.A. Berzofsky and I.J. Berkower, in Fundamental Immunology (ed. W.E. Paul), Raven Press (New York), 595 (1984), which is incorporated herein by reference). The humanized NA-8 antibodies had an affinity within about 2-fold of the mouse NA-8 antibody. A similar result will be found when the affinity for CD18 on human neutrophils is measured. The humanized NA-8a antibody was similarly determined to have approximately the same binding affinity for CD18 as humanized NA-8.

The ability of the mouse and humanized NA-8 antibodies to block the adhesion of human neutrophils to endothelial cells was shown as follows (Fig 5). Human umbilical cord endothelial cells (HUVEC; from Clonetics, San Diego) were grown to confluency in EGM media (Clonetics) in a 24 well plate. Four hours prior to the assay, each well was washed with RPMI, and media containing 20 ng/ml IL-1β was added. The plates were then incubated at 37° C. Human neutrophils were isolated from buffy coats that had been cleared of erythrocytes by dextran sedimentation, and then adjusted to 10⁷ cells per ml. The neutrophils were then labelled with 200-400 μCi of ⁵¹Cr. Twenty minutes prior to the assay, 250 μl of the labelled neutrophil suspension was added to a polypropylene tube containing 250 μl of RPMI with varying

concentrations of an antibody. The assay was begun by washing the HUVEC free of IL-1 β and adding antibody-treated cells to the wells. The plate was incubated for thirty minutes at 37° C to allow the neutrophils to settle onto the activated HUVEC. Each well was washed 3 times with RPMI to remove non-adherent neutrophils. The contents of each well was lysed by the addition of 500 μ l of 1% SDS, and transferred to a vial to determine adherent cpm. The humanized NA-8a antibody similarly blocked binding of neutrophils to HUVEC.

From the foregoing, it will be appreciated that the humanized immunoglobulins of the present invention offer numerous advantages over other CD18 specific antibodies. In comparison to mouse monoclonal antibodies, the present humanized immunoglobulins can be more economically produced and contain substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WE CLAIM:

1. A humanized immunoglobulin having complementarity determining regions (CDRs) corresponding to CDRs from the donor immunoglobulin NA-8 and heavy and light chain variable region frameworks corresponding to human acceptor immunoglobulin heavy and light chain frameworks, which humanized immunoglobulin specifically binds to human CD18 with an affinity constant of at least 10^7 M^{-1} , wherein the sequence of the humanized immunoglobulin heavy chain variable region framework is more than 65% identical but less than 95 % identical to the sequence of the donor immunoglobulin heavy chain variable region framework.

2. A humanized immunoglobulin according to claim 1 which is an antibody comprising two light chain/heavy chain dimers.

3. A humanized immunoglobulin of claim 2, wherein said antibody is of the IgG1 or IgG4 isotype.

4. A humanized immunoglobulin according to claim 1, which specifically binds to human CD18 with an affinity of at least 10^8 M^{-1} .

5. A humanized immunoglobulin according to claim 1, which is an Fab or (Fab')₂.

6. A humanized immunoglobulin according to claim 1, wherein said acceptor immunoglobulin heavy and light chain frameworks are from the same human antibody.

7. A humanized immunoglobulin according to claim 6, wherein said human antibody is the Eu human antibody.

8. A humanized immunoglobulin having complementarity determining regions (CDRs) corresponding to CDRs from the donor immunoglobulin NA-8 and heavy and light

chain variable region frameworks corresponding to acceptor immunoglobulin heavy and light chain frameworks, which humanized immunoglobulin specifically binds to human CD18 with an affinity constant of at least 10^7 M^{-1} , wherein the sequence of the acceptor immunoglobulin heavy chain variable region is among the 5 sequences in a representative collection of sequences of human immunoglobulin heavy chain variable regions most homologous to the sequence of the donor immunoglobulin heavy chain variable region.

9. A humanized immunoglobulin having complementarity determining regions (CDRs) corresponding to CDRs from the donor immunoglobulin NA-8 and heavy and light chain variable region frameworks corresponding to acceptor immunoglobulin heavy and light chain frameworks, which humanized immunoglobulin specifically binds to human CD18 with an affinity constant of at least 10^7 M^{-1} , wherein said humanized immunoglobulin comprises amino acids from the donor immunoglobulin framework replacing the corresponding amino acids in the acceptor immunoglobulin heavy or light chain frameworks, said amino acids not in positions 26-30 of the heavy chain, and each of said amino acids:

- (i) is adjacent to a CDR in the donor immunoglobulin sequence, or
- (ii) contains an atom within a distance of 5 angstroms of a CDR in said humanized immunoglobulin.

10. A humanized immunoglobulin having complementarity determining regions (CDRs) corresponding to CDRs from the donor immunoglobulin NA-8 and heavy and light chain variable region frameworks corresponding to acceptor immunoglobulin heavy and light chain frameworks, which humanized immunoglobulin specifically binds to human CD18 with an affinity constant of at least 10^7 M^{-1} , wherein said humanized immunoglobulin comprises amino acids from the donor immunoglobulin framework replacing the corresponding amino acids in the acceptor immunoglobulin heavy or light chain

frameworks, said amino acids not in positions 26-30 of the heavy chain, and each of said amino acids:

- (i) is adjacent to a CDR in the donor immunoglobulin sequence, or
- (ii) contains an atom within a distance of 4 angstroms of a CDR in said humanized immunoglobulin.

11. A humanized immunoglobulin according to claims 9 or 10 wherein the distance from said atom to said CDR is determined from a computer-generated model of an immunoglobulin.

12. A humanized immunoglobulin according to claims 9 or 10, which is an Fab or (Fab')₂.

13. A humanized immunoglobulin according to claim 9 which is an antibody comprising two light chain/heavy chain dimers.

14. A humanized immunoglobulin of claim 13, wherein said antibody is of the IgG1 or IgG4 isotype.

15. A humanized immunoglobulin according to claim 9, wherein said acceptor immunoglobulin heavy and light chain frameworks are both from the Eu human antibody.

16. A humanized immunoglobulin according to claims 1 or 9 which is substantially pure.

17. A humanized immunoglobulin according to claims 1 or 9 that inhibits the binding of human neutrophils to human endothelial cells.

18. A composition comprising a humanized immunoglobulin according to claims 1 or 9.

19. A recombinant immunoglobulin which specifically binds to human CD18, wherein the amino acid sequence of the

mature light chain variable region is as shown in the lower lines of Fig. 2A.

5 20. A recombinant immunoglobulin which specifically binds to human CD18, wherein the amino acid sequence of the mature heavy chain variable region is as shown in the lower lines of Fig. 2B or Fig. 2C.

10 21. A method of treating an inflammatory disease or condition, comprising administering to a human patient a therapeutically-effective dose of a humanized immunoglobulin which specifically binds to human CD18.

15 22. A method according to claim 21, wherein the inflammatory disease or condition is selected from the group consisting of: ischemia-reperfusion injury, myocardial infarction, balloon angioplasty, cardiac surgery, adult respiratory distress syndrome, cerebral ischemia, sepsis, and autoimmune disease.

20 23. A method according to claim 21, wherein the humanized immunoglobulin comprises the amino acid sequence of the mature light chain variable region as shown in the lower lines of Fig. 2A and the amino acid sequence of the mature heavy chain variable region as shown in the lower lines of Fig. 2B or Fig. 2C.

25 24. A method according to claim 21, wherein the humanized immunoglobulin binds to human CD18 with an affinity of at least $1 \times 10^7 \text{ M}^{-1}$.

30 25. A method according to claim 24, wherein at least about 10 mg of the humanized immunoglobulin is administered by a parenteral route.

35 26. The murine antibody NA-8.

27. A cell line producing an antibody of claim 26.

28. An immunoglobulin whose light or heavy chain variable region respectively comprises the CDRs shown in Fig. 2A or Fig. 2B.

5 29. An immunoglobulin according to claim 28 which is humanized.

 30. An immunoglobulin according to claim 29 which is an antibody comprising two light chain/heavy chain dimers.
10

1 / 6

30 60
 ATGGATTCA[.]CAGGCC[.]CAGGTTCTTATGTTACTGCTGCTATGGGTATCTGGAACCTGTGGG
 M D S Q A Q V L M L L L W V S G T C G
 90 120
 GACATTGTGATGTCACAGTCTCCATCCTCCCTAGCTGTGTCAGTTGGAGAGAAGGTTACT
 D I V M S Q S P S S L A V S V G E K V T
 150 180
 ATGAGCTGCAAGTCCAGTCAGAGCCTTTTATATAGTAGCATTCAAAGAATTACTTGGCC
 M S C K S S Q S L L Y S S I Q K N Y L A
 210 240
 TGGTACCAGCAGAAACCAGGGCAGTCTCCTAAACTGCTGATTTACTGGGCATCTACTAGG
 W Y Q Q K P G Q S P K L L I Y W A S T R
 270 300
 GAATCTGGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACC
 E S G V P D R F T G S G S G T D F T L T
 330 360
 ATCAGCAGTGTGAAGGCTGAAGACCTGGCAGTTTATTACTGTCAGCAATATTATAGCTAT
 I S S V K A E D L A V Y Y C Q Q Y Y S Y
 390
 CCATTACGTTTCGGCTCGGGGACAAAGTTGGAATAAAA
 P F T F G S G T K L E I K

Fig. 1A

30 60
 ATGGAAAGGCACTGGATCTTTCTCTTCCTGTTTTAGTA[.]ACTGCAGGTGTCCACTCCCAG
 M E R H W I F L F L F S V T A G V H S Q
 90 120
 GTCCAGCTTCAGCAGTCTGGGGCTGA[.]ACTGGCAAACCTGGGGCCTCAGTGAAGATGTCC
 V Q L Q Q S G A E L A K P G A S V K M S
 150 180
 TGCAAGGCTTCTGGCTACACCTTTACTAGTTACTTGATGCACTGGGTAA[.]ACAGAGGCCT
 C K A S G Y T F T S Y L M H W V K Q R P
 210 240
 GGACAGGGTCTGGAATGGATTGGAACATTAACTCCTAGCACTGCTTATA[.]CCGACTACAAT
 G Q G L E W I G N I N P S T A Y T D Y N
 270 300
 CAGAACTTTA[.]ACGACAAGGCCACATTGACTGCAGACAGATCCTCCACCACAGCCTACATG
 Q N F N D K A T L T A D R S S T T A Y M
 330 360
 CAACTGAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAGGGGGTGAT
 Q L S S L T S E D S A V Y Y C A R G G D
 390
 TTCCTTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
 F L M D Y W G Q G T S V T V S S

Fig. 1B

1	D	I	V	M	S	Q	S	P	S	S	L	A	V	S	V	G	E	K	V	T
1	D	I	Q	M	T	Q	S	P	S	T	L	S	A	S	V	G	D	R	V	T
21	M	S	C	K	S	S	Q	S	L	L	Y	S	S	I	Q	K	N	Y	L	A
21	I	<u>S</u>	C	K	S	S	Q	S	L	L	Y	S	S	I	Q	K	N	Y	L	A
41	W	Y	Q	Q	K	P	G	Q	S	P	K	L	L	I	Y	W	A	S	T	R
41	W	Y	Q	Q	K	P	G	K	<u>S</u>	P	K	L	L	<u>I</u>	Y	W	A	S	T	R
61	E	S	G	V	P	D	R	F	T	G	S	G	S	G	T	D	F	T	L	T
61	<u>E</u>	<u>S</u>	G	V	P	S	R	F	<u>T</u>	G	S	G	S	G	T	<u>D</u>	F	T	L	T
81	I	S	S	V	K	A	E	D	L	A	V	Y	Y	C	Q	Q	Y	Y	S	Y
81	I	S	S	L	Q	P	<u>E</u>	D	F	A	T	Y	Y	C	<u>Q</u>	<u>Q</u>	<u>Y</u>	<u>Y</u>	<u>S</u>	<u>Y</u>
101	P	F	T	F	G	S	G	T	K	L	E	I	K							
101	<u>P</u>	<u>F</u>	<u>T</u>	<u>F</u>	<u>G</u>	<u>Q</u>	<u>G</u>	<u>T</u>	<u>K</u>	<u>V</u>	<u>E</u>	<u>I</u>	<u>K</u>							

Fig 2A

1	Q	V	Q	L	Q	Q	S	G	A	E	L	A	K	P	G	A	S	V	K	M
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	A	S	G	Y	T	F	T	S	Y	L	M	H	W	V	K	Q	R
21	S	C	K	A	S	G	<u>Y</u>	T	F	<u>T</u>	<u>S</u>	<u>Y</u>	<u>L</u>	<u>M</u>	<u>H</u>	W	V	<u>K</u>	<u>Q</u>	A
41	P	G	Q	G	L	E	W	I	G	N	I	N	P	S	T	A	Y	T	D	Y
41	P	G	Q	G	L	E	W	<u>I</u>	G	<u>N</u>	<u>I</u>	<u>N</u>	<u>P</u>	<u>S</u>	<u>T</u>	A	Y	T	D	Y
61	N	Q	N	F	N	D	K	A	T	L	T	A	D	R	S	S	T	T	A	Y
61	<u>N</u>	<u>Q</u>	<u>N</u>	<u>F</u>	<u>N</u>	<u>D</u>	<u>K</u>	<u>A</u>	<u>T</u>	<u>L</u>	<u>T</u>	<u>A</u>	<u>D</u>	<u>R</u>	<u>S</u>	T	N	T	A	Y
81	M	Q	L	S	S	L	T	S	E	D	S	A	V	Y	Y	C	A	R	G	G
81	M	E	L	S	S	L	R	S	E	D	T	A	<u>V</u>	<u>Y</u>	<u>Y</u>	C	A	<u>R</u>	<u>G</u>	<u>G</u>
101	D	F	L	M	D	Y	W	G	Q	G	T	S	V	T	V	S	S			
101	<u>D</u>	<u>F</u>	<u>L</u>	<u>M</u>	<u>D</u>	<u>Y</u>	<u>W</u>	<u>G</u>	<u>Q</u>	<u>G</u>	<u>T</u>	<u>L</u>	<u>V</u>	<u>T</u>	<u>V</u>	<u>S</u>	<u>S</u>			

Fig. 2B

3 / 6

1	Q	V	Q	L	Q	Q	S	G	A	E	L	A	K	P	G	A	S	V	K	M
1	Q	V	Q	L	V	Q	S	G	A	E	V	K	K	P	G	S	S	V	K	V
21	S	C	K	A	S	G	Y	T	F	T	S	Y	L	M	H	W	V	K	Q	R
21	S	C	K	A	S	G	<u>Y</u>	T	F	<u>T</u>	<u>S</u>	<u>Y</u>	<u>L</u>	<u>M</u>	<u>H</u>	W	V	<u>K</u>	Q	A
41	P	G	Q	G	L	E	W	I	G	N	I	N	P	S	T	A	Y	T	D	Y
41	P	G	Q	G	L	E	W	<u>I</u>	G	<u>N</u>	<u>I</u>	<u>N</u>	<u>P</u>	<u>S</u>	<u>T</u>	<u>A</u>	<u>Y</u>	<u>T</u>	<u>D</u>	<u>Y</u>
61	N	Q	N	F	N	D	K	A	T	L	T	A	D	R	S	S	T	T	A	Y
61	<u>N</u>	<u>Q</u>	<u>N</u>	<u>F</u>	<u>N</u>	<u>D</u>	R	V	T	I	T	A	D	E	S	T	N	T	A	Y
81	M	Q	L	S	S	L	T	S	E	D	S	A	V	Y	Y	C	A	R	G	G
81	M	E	L	S	S	L	R	S	E	D	T	A	<u>V</u>	Y	<u>Y</u>	C	A	<u>R</u>	<u>G</u>	<u>G</u>
101	D	F	L	M	D	Y	W	G	Q	G	T	S	V	T	V	S	S			
101	<u>D</u>	<u>F</u>	<u>L</u>	<u>M</u>	<u>D</u>	<u>Y</u>	<u>W</u>	<u>G</u>	<u>Q</u>	<u>G</u>	<u>T</u>	L	V	T	V	S	S			

Fig. 2C

```

      10      20      30      40      50      60
TCTAGACCACCATGGAGAAAGACACACTCCTGCTATGGGTCCTGCTTCTCTGGGTTCCAG
      M E K D T L L L W V L L L W V P

      70      80      90      100     110     120
GTTCCACAGGTGATATACAGATGACTCAGTCACCGTCGACTCTGAGTGCTTCAGTCGGTG
      G S T G D I Q M T Q S P S T L S A S V G

      130     140     150     160     170     180
ATCGTGTCACTATATCATGTAAGAGCTCACAGTCACTACTATATTCTAGTATACAGAAGA
      D R V T I S C K S S Q S L L Y S S I Q K

      190     200     210     220     230     240
ACTATCTAGCTTGGTATCAGCAGAAGCCTGGTAAGTCACCTAAGCTTCTGATCTACTGGG
      N Y L A W Y Q Q K P G K S P K L L I Y W

      250     260     270     280     290     300
CGAGTACACGTGAGTCTGGAGTACCAAGTCGTTTCACAGGTAGTGGCTCAGGCACGGATT
      A S T R E S G V P S R F T G S G S G T D

      310     320     330     340     350     360
TCACACTGACTATATCGTCACTGCAGCCAGAGGACTTCGCAACGTACTACTGTCAGCAGT
      F T L T I S S L Q P E D F A T Y Y C Q Q

      370     380     390     400     410     420
ATTACTCATATCCGTTACGTTCCGACAGGGAAGTAAGGTAGAGATCAAGCGTAAGTAGA
      Y Y S Y P F T F G Q G T K V E I K

      430
ATCCAAAGTCTAGA

```

Fig. 3A

```

      10      20      30      40      50      60
TCTAGACCACCATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGAACTGCTGGCG
      M G W S W I F L F L L S G T A G

      70      80      90      100     110     120
TCCACTCTCAAGTACAGCTAGTGCAGTCTGGAGCTGAGGTCAAGAAGCCTGGATCGTCAG
      V H S Q V Q L V Q S G A E V K K P G S S

      130     140     150     160     170     180
TCAAGGTCAGCTGTAAGGCATCAGGATATACGTTACGTCATACCTGATGCACTGGGTGA
      V K V S C K A S G Y T F T S Y L M H W V

      190     200     210     220     230     240
AGCAGGCCCTGGACAGGGACTCGAGTGGATCGGTAACATAAATCCGTCGACTGCATATA
      K Q A P G Q G L E W I G N I N P S T A Y

      250     260     270     280     290     300
CGGACTATAATCAGAATTTCAACGATAAGGCAACTCTGACGGCTGATCGATCGACAAACA
      T D Y N Q N F N D K A T L T A D R S T N

      310     320     330     340     350     360
CTGCGTATATGGAGCTGTCAAGCTTGCGATCAGAAGATACTGCTGTTTACTACTGCGCAC
      T A Y M E L S S L R S E D T A V Y Y C A

      370     380     390     400     410     420
GAGGTGGAGACTTCCTGATGGACTATTGGGACAGGGTACGCTAGTAACGGTGAGCTCAG
      R G G D F L M D Y W G Q G T L V T V S S

      430
GTAAGAATGGCCTCTAGA

```

Fig. 3B

5 / 6

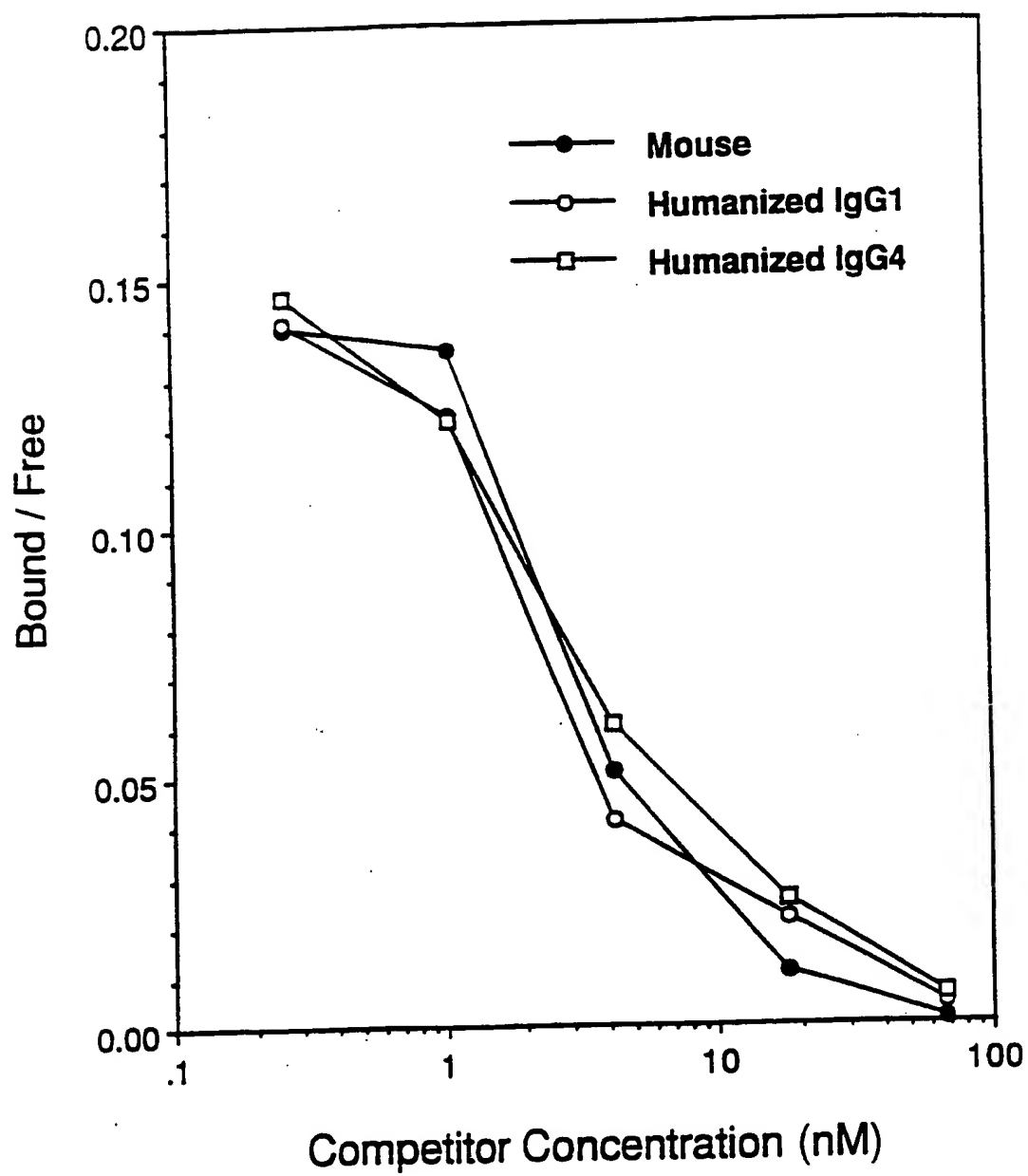


Figure 4

6 / 6

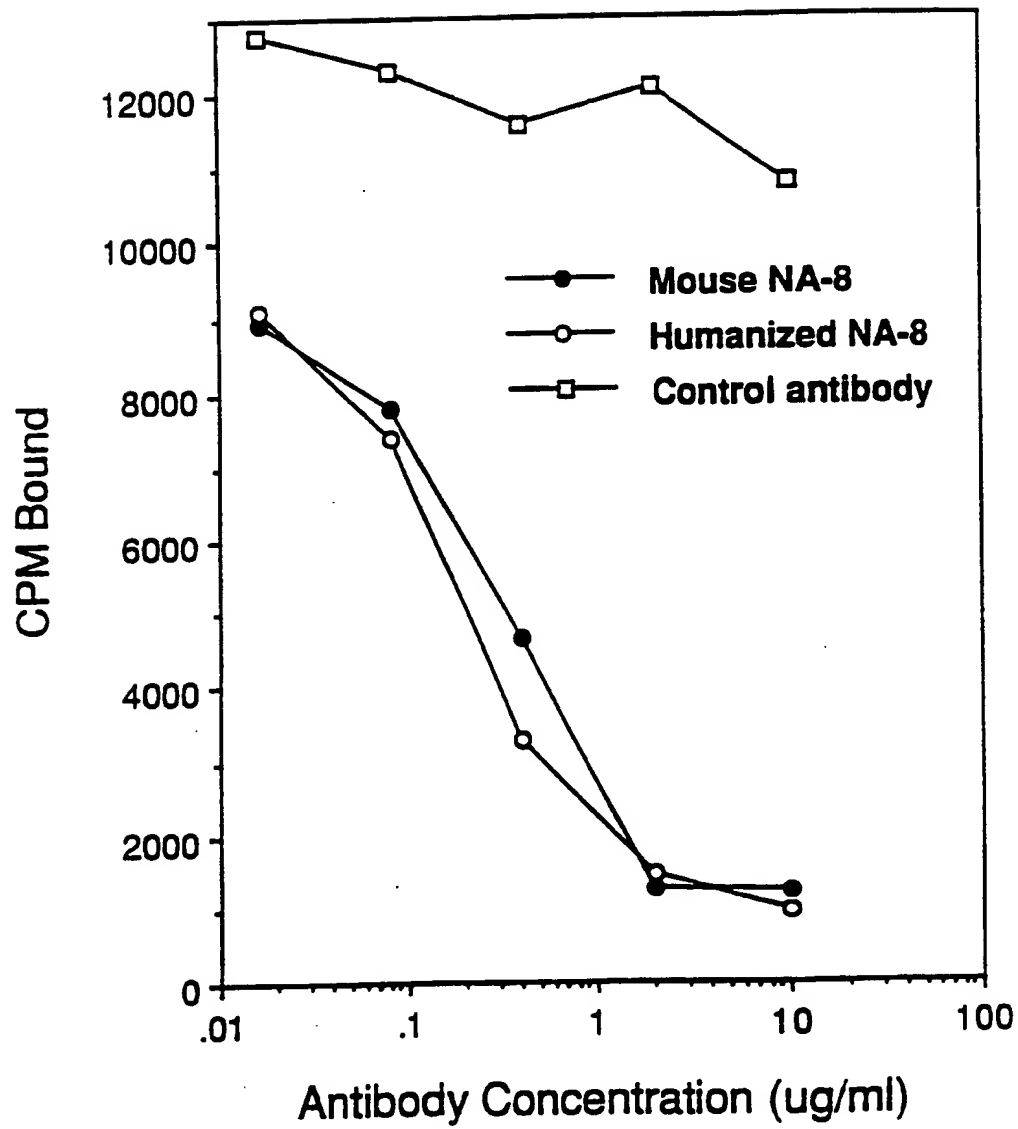


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/11611

A. CLASSIFICATION OF SUBJECT MATTER				
IPC(5) :A61K 39/395; C07K 15/28; C12N 15/13; C12P 21/08 US CL :424/85.8; 530/387.1, 387.3, 388.1, 388.22, 388.7 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED				
Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/85.8; 530/387.1, 387.3, 388.1, 388.22, 388.7				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, DIALOG, BIOSIS, EMBASE, MEDLINE, WPI search terms: CD18, humanized, chimeric, antibody, NA-8, Co, Landolfi				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y,P	TIBTECH, Volume 11, issued February 1993, W.J. Harris et al., "Therapeutic Antibodies -The Coming of Age", pages 42-45, see entire document.	1-30		
Y	Immunol. Reviews, Volume 114, issued April 1990, M. Arnaout, "Leukocyte Adhesion Molecules Deficiency: Its Structural Basis, Pathophysiology and Implications for Modulating the Inflammatory Response", pages 145-179, see entire document.	1-30		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.				
<table border="0"> <tr> <td> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be part of particular relevance</p> <p>"E" earlier document published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be part of particular relevance</p> <p>"E" earlier document published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be part of particular relevance</p> <p>"E" earlier document published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>			
Date of the actual completion of the international search 13 January 1994		Date of mailing of the international search report FEB 17 1994		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE		Authorized officer PHILLIP GAMBEL <i>P. Kuyza</i> Telephone No. (703) 308-0196		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/11611

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci., Volume 86, issued December 1989, C. Queen et al., "A Humanized Antibody That Binds To The Interleukin 2 Receptor", pages 10029-10033, see entire document.	1-30
Y	EP,A,0,440,351 (Law et al.) 07 August 1991, see entire document	1-30
Y	US, A, 5,147,637 (Wright et al.) 15 September 1992, see entire document.	1-30
Y	Proc. Natl. Acad. Sci., Volume 80, issued September 1983, S.D. Wright et al., "Identification of the C3bi Receptor of Human Monocytes and Macrophages by Using Monoclonal Antibodies", pages 5699-5703, see entire document.	1-30
Y	Nucleic Acids Res., Volume 19, No. 9 issued 1991, B.L. Daugherty et al., "Polymerase Chain Reaction Facilitates the Cloning, CDR-Grafting, and Rapid Expression of a Murine Monoclonal Antibody Directed Against the CD18 Component of Leukocyte Integrins", pages 2471-2476, see entire document.	1-30